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ORIGINAL CONTRIBUTION

Exhibit M

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Acute cardioprotective effects of erythropoietin in infant rabbits are mediated by activation of protein kinases and potassium channels

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Abstract Erythropoietin is protective against cardiac ischemia, but the underlying mechanisms are unknown. We determined whether erythropoietin (0.5 – 10.0 U/ml) confers acute cardioprotection in infant rabbit hearts and the contribution of protein kinases, nitric oxide synthase and potassium channels to the underlying mechanism. Hearts from normoxic infant New Zealand White rabbits (n=8/group) were isolated and perfused in the Langendorff mode. Biventricular function was recorded under steady-state conditions prior to 30 min global no-flow ischemia and 35 min reperfusion. Administration of erythropoietin for 15 min immediately prior to ischemia resulted in a concentration-dependent increase in recovery of left and right ventricular developed pressure in rabbit hearts following myocardial ischemia and reperfusion. The optimal concentration of erythropoietin that afforded maximum recovery of developed pressure was manifest at 1.0 U/ml. Erythropoietin (1.0 U/ml) treatment resulted in phosphorylation of PKC_ε, p38 MAP kinase and p42/44 MAP kinase. The cardioprotective effects of erythropoietin were abolished by the protein kinase inhibitors SB203580 (p38 MAP kinase), PD98059 (p42/44 MAP kinase) and chelerythrine (PKC) as well as the potassium channel blockers glibenclamide, HMR 1098, 5-HD and Paxilline. Nitrite and nitrate release from hearts before (2.3 ± 0.9 nmol/min/g) and after (2.4 ± 1.9 nmol/min/g) 15 min treatment with erythropoietin (1.0 U/ml) were not different. L-NAME and L-NMA did not block the cardioprotective effect of erythropoietin. We conclude the rapid activation of potassium channels and protein kinases by erythropoietin represents an important new mechanism for increasing cardioprotection.

Keywords Ischemia – molecular biology – erythropoietin – protein kinases – potassium channels

Introduction

Congenital heart defects occur in one out of every 125 newborn children [13]. One third of these children require a major surgical procedure within the first year of life to prevent premature death. Many of these children exhibit varying degrees of cyanosis where the myocardium is chronically perfused with hypoxic blood. Understanding the mechanisms by which cyanotic congenital heart defects modify the myocardium may provide insights into developing treatments to protect the hearts from these children during corrective surgery.

To investigate the effects of chronic hypoxia on signal transduction mechanisms we developed an animal model in which rabbits are raised from birth in a hypoxic

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environment [2]. Recently we showed that infant human and rabbit hearts adapt to chronic hypoxemia by activation of PKC_ε, p38 MAP kinase and JUN kinase signaling pathways [17] and by increasing nitric oxide production [19]. Activation of these protein kinase-signaling pathways and nitric oxide synthase in infant hearts adapted to chronic hypoxia is associated with increased resistance to ischemia, which is mediated by ATP-sensitive K^+ (K_{ATP}) channels [3].

Chronic hypoxia from birth also results in erythropoiesis as manifest by an increase in hemoglobin and hematocrit [2]. Erythropoietin activates protein kinase signaling pathways [22] and can increase resistance to cerebral ischemia [21]. Recently erythropoietin has been observed to increase resistance of the heart to regional ischemia *in vivo* [6]. However the signal transduction pathway involved and the end effectors mediating cardioprotection were not examined. The role of erythropoietin in acute cardioprotection in the setting of cardiac surgery, where the heart is subjected to global ischemia, is unknown. To determine a possible role for erythropoietin in cardioprotection during surgical ischemia and the underlying mechanisms we treated infant rabbit hearts with human recombinant erythropoietin prior to ischemia. The objectives of our study were to determine whether acute exposure of the heart to erythropoietin would increase resistance to subsequent ischemia, the erythropoietin concentration that confers optimal protection of the heart, the involvement and cellular location of protein kinase signaling pathways, and the role of potassium channels and nitric oxide synthase in mediating cardioprotection.

Methods

Animals

Rabbits used in this study received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" formulated by the National Research Council, 1996. Infant New Zealand White rabbits were maintained for 10 days in a normoxic ($SaO_2 > 95\%$) or hypoxic ($SaO_2 < 85\%$) environment from birth as described previously [4].

Reagents

Recombinant human erythropoietin was obtained from Cell Science, Inc. (Norwood, MA). Glibenclamide was obtained from Calbiochem (San Diego, CA). 5-HD was purchased from Sigma-Aldrich (St. Louis, MO) with HMR 1098 kindly provided by Dr. Garrett Gross. Chelerythrine, PD98059 and SB203580 were obtained from Sigma-Aldrich (St. Louis, MO), Biomol Research Laboratories, Inc. (Plymouth Meeting, PA) and Calbiochem (San Diego, CA), respectively. Paxilline was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Antibodies to phosphorylated and non-phosphorylated p44/42 MAP kinase and p38 MAP kinase were obtained from Cell Signaling Tech (Beverly, MA). Anti PKC_ε was obtained from Calbiochem (San Diego, CA) and anti phospho PKC_ε was obtained from Upstate Biotech, Inc. (Lake Placid, NY). The secondary antibody was horseradish peroxidase obtained from Zymed (South San Francisco, CA).

Isolated heart perfusion

Isolated rabbit hearts were perfused with bicarbonate buffer at constant pressure in a retrograde manner. Protein kinase inhibitors, potassium channel blockers or nitric oxide synthase inhibitors were added to this perfusate as needed. A 3-way tap, located immediately above the site of cannulation, allowed the entire perfusate to be diverted away from the heart to produce global, no-flow ischemia. Reperfusion was achieved by repositioning of the tap to allow perfusate to be delivered to the heart. Left and right ventricular function was monitored continuously throughout each experiment as previously described [4]. End-diastolic pressure was initially set to 3 mmHg for 2 minutes. The balloons were then progressively inflated with a microsyringe to set end-diastolic pressures to 8 mmHg for the left ventricle and 4 mmHg for the right ventricle, with developed pressure and heart rate recorded during steady-state conditions. Coronary flow rate was measured throughout the experiment by timed collections of the coronary effluent from the right side of the heart into a graduated cylinder. Coronary flow rate was expressed as milliliters per minute per gram wet weight.

Resistance to myocardial ischemia

Hearts from infant rabbits were perfused with bicarbonate buffer, and biventricular function was monitored continuously throughout each experiment as previously described [17]. For concentration response studies, hearts were then perfused with erythropoietin (0.5 – 10.0 U/ml) for 15 minutes prior to 30 minutes ischemia and 35 minutes reperfusion. The experimental protocol used is shown in Fig. 1. For mechanism studies with protein kinase inhibitors, potassium channel blockers or nitric oxide synthase inhibitors, hearts were perfused with drugs for 15 minutes alone followed by 15 minutes in combination with erythropoietin prior to ischemia. Hearts perfused with protein kinase inhibitors or potas-

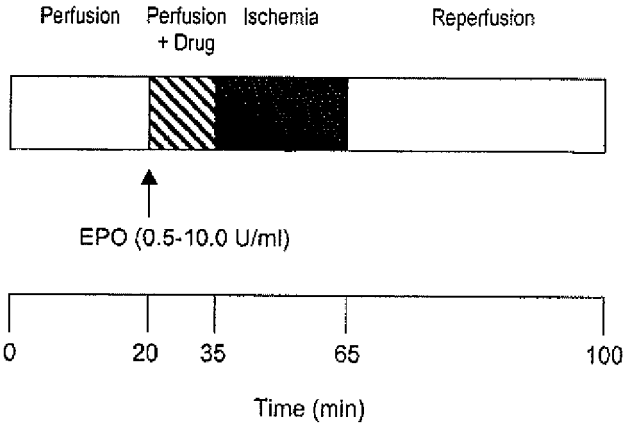

Fig. 1 Experimental protocol used for the erythropoietin concentration response studies

sium channel blockers alone in the absence of erythropoietin for 30 minutes prior to ischemia served as untreated controls for these studies. Recovery of post-ischemic left and right ventricular developed pressure was expressed as a percentage of its pre-drug, pre-ischemic value.

Western analysis

Hearts from infant rabbits were isolated and aerobically perfused with bicarbonate buffer for 20 minutes, then perfused with erythropoietin for 5 or 15 minutes. The free wall of the left ventricle was excised and immediately freeze-clamped between stainless steel tongs pre-cooled with liquid nitrogen. Frozen myocardial tissue samples were powdered in a pre-cooled stainless steel mortar and pestle. Powdered tissue was homogenized in sample buffer (50 mM Tris pH 7.5, 5 mM EDTA, 10 mM EGTA, 10 mM benzamidine, 10 μg/ml pepstatin A, 50 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 0.3% β-mercaptoenthanol) on ice for 50 strokes. Nuclei and cellular debris was removed by centrifugation (1000 g at 4 °C for 15 min). The supernatant was transferred to a new cold 1.5 mL microcentrifuge tube. The cytosolic and particulate portions of total cellular proteins were separated by a 30-minute centrifugation at 45000 g. Protein concentrations were determined by the method of Bradford. Equal amounts of protein were analyzed by SDS-PAGE and Western blotting by using either isoform-specific antibodies for phospho-PKC detection or specific antibodies against phosphorylated and nonphsophorylated p38 MAPK, JNK, and p42/44 MAPK. The blots were developed by ECL. Densitometry was performed on each sample and analyzed with the use of NIH image software [17].

Erythropoietin analysis

Venous blood was withdrawn from normoxic and chronically hypoxic infant rabbits (n = 5/group). The serum was analyzed for erythropoietin concentration using a standard immunochemiluminometric assay (Quest Diagnostics, San Juan Capistrano, CA).

Table 1 Hemodynamic values for erythropoietin concentration-response studies in normoxic hearts and cardioprotection studies in chronically hypoxic hearts (see Figs. 2 and 7)

	PRE DRUG				POST DRUG				REPERFUSION (35 min)			
Groups	Heart rate (beats/min)	Coronary flow rate (ml/min/g)	Left ventricle developed pressure (mmHg)	Right ventricle developed pressure (mmHg)	Heart rate (beats/min)	Coronary flow rate (ml/min/g)	Left ventricle developed pressure (mmHg)	Right ventricle developed pressure (mmHg)	Heart rate (beats/min)	Coronary flow rate (ml/min/g)	Left ventricle developed pressure (mmHg)	Right ventricle developed pressure (mmHg)
1. Normoxia (N)	244 ± 19	5 ± 1	105 ± 9	42 ± 7					245 ± 23	4 ± 1	52 ± 4	28 ± 4
2. N+EPO (0.5 U/ml)	223 ± 15	6 ± 1	106 ± 6	41 ± 4	196 ± 21	3 ± 1	102 ± 8	38 ± 5	210 ± 19	4 ± 1	67 ± 3	31 ± 4
3. N+EPO (1.0 U/ml)	229 ± 16	6 ± 1	101 ± 3	40 ± 3	199 ± 25	3 ± 1	99 ± 4	38 ± 3	210 ± 30	5 ± 1	71 ± 8	31 ± 2
4. N+EPO (2.5 U/ml)	226 ± 14	6 ± 1	101 ± 3	42 ± 4	210 ± 20	4 ± 1	103 ± 4	41 ± 5	224 ± 14	5 ± 2	64 ± 3	31 ± 3
5. N+EPO (5.0 U/ml)	236 ± 23	6 ± 1	101 ± 3	43 ± 6	221 ± 20	4 ± 1	102 ± 6	43 ± 7	226 ± 26	4 ± 1	64 ± 4	32 ± 5
6. N+EPO (10 U/ml)	248 ± 37	6 ± 1	103 ± 3	42 ± 3	232 ± 52	5 ± 2	101 ± 5	40 ± 5	230 ± 49	5 ± 1	52 ± 3	28 ± 4
7. Hypoxia	194 ± 10	6 ± 1	100 ± 8	49 ± 7					207 ± 29	5 ± 1	65 ± 4	38 ± 6
8. Hypoxia + EPO (1.0 U/ml)	198 ± 19	6 ± 1	105 ± 10	48 ± 7	181 ± 16	5 ± 1	117 ± 15	46 ± 8	204 ± 25	6 ± 2	77 ± 7	42 ± 6

EPO = erythropoietin

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Statistical analysis

Data reported are mean ± SD. Statistical analysis was performed by use of repeated measures ANOVA with the Greenhouse-Geisser adjustment used to correct for the inflated risk of a Type I error [4]. If significant, the Mann-Whitney test was used as a second step to identify which groups were significantly different. After ANOVA the data were analyzed for differences related to multiple comparisons [4]. Significance was set at $P < 0.05$.

Results

Erythropoietin concentration-response studies

Erythropoietin protects the brain against ischemic damage [21] by a mechanism involving protein kinase signaling. As these pathways also protect the heart against ischemic damage [17] we reasoned erythropoietin might also confer acute cardioprotection. Hearts from normoxic New Zealand White rabbits at 10 days of age were perfused with erythropoietin at 0.5, 1.0, 2.5, 5.0, and 10.0 U/ml for 15 minutes prior to 30 minutes global ischemia and 35 minutes reperfusion. Erythropoietin (1.0 U/ml) reduced coronary flow rate prior to ischemia from 6 ml/min/g to 3 ml/min/g but had no effect on heart rate (199 ± 25 beats/min) or developed pressure in left (99 ± 4 mmHg) or right (38 ± 3 mmHg) ventricle (Table 1). Erythropoietin increased recovery of left and right ventricular developed pressure following ischemia and reperfusion in a bell-shaped concentration-dependent manner. The optimal concentration that afforded maximal recovery of post-ischemic left and right ventricular developed pressure was manifested at 1.0 U/ml (Fig. 2). Recovery of coronary flow rate was also increased from 75 ± 2% in untreated hearts to 86 ± 2% of pre-ischemic values in hearts treated with 1.0 U/ml erythropoietin. Recovery of heart rate was unaffected by erythropoietin. To determine the time-dependency of cardioprotection hearts were perfused for 5 minutes with erythropoietin prior to ischemia. Treatment of hearts for 5 minutes with erythropoietin at the optimal dose of 1.0 U/ml prior to ischemia increased post-ischemic recovery of right ventricular developed pressure from 68 ± 7% to 82 ± 12%. Erythropoietin (1.0 U/ml) treatment for 5 minutes prior to ischemia had no effect on recovery of post-ischemic left ventricular developed pressure (52 ± 6%) compared with untreated controls (49 ± 2%). These data indicate erythropoietin acutely protects the heart against ischemic injury in a concentration- and time-dependent manner.

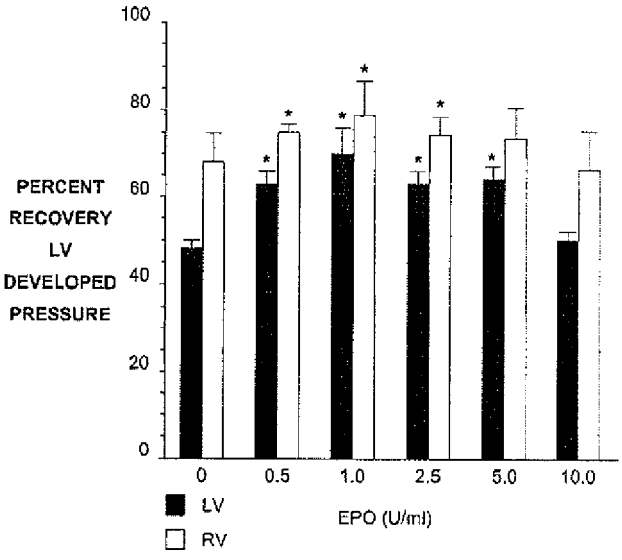

Fig. 2 Erythropoietin concentration-response studies. Recovery of left and right ventricular developed pressure in heart following 15 min treatment with erythropoietin (0.5, 1.0, 2.5, 5.0, and 10.0 U/ml) prior to 30 min global ischemia and 35 min reperfusion. Data are means * SD, $n = 8$ hearts/group. * = $P < 0.05$, EPO vs. drug-free control

Role of protein kinases in erythropoietin-induced cardioprotection

Since binding of erythropoietin to the erythropoietin receptor activates protein kinase signaling pathways, we sought to identify the downstream pathways that underlie cardioprotection conferred by erythropoietin. We used the concentration of erythropoietin that was found to confer optimal cardioprotection (1.0 U/ml). Hearts from normoxic rabbits were perfused with protein kinase inhibitors alone for 15 minutes and then combined with erythropoietin for a further 15 minute period prior to ischemia. Inhibitors of PKC (chelerythrine), p38 MAPK (SB203580) and p42/44 MAPK (PD98059) used at concentrations previously found to block the cardioprotective effect of hypoxia [17] all abolished the cardioprotective effect of erythropoietin (Fig. 3). There was no effect of these inhibitors on cardioprotection in control hearts indicating these protein kinases are not active in untreated hearts (Fig. 3). Since cardioprotection by erythropoietin is regulated by inhibitors of protein kinases, we determined if erythropoietin treatment of hearts resulted in phosphorylation of these protein kinases by Western blot analysis using monoclonal antibodies specific for PKC_ε, phosphorylated p38 MAP kinase (Thr180/Tyr182) and p42/44 MAP kinase (Thr202/Tyr204). Non-phosphorylated antibodies were used to ensure equal loading of proteins. Analysis of the cytosolic and partic-

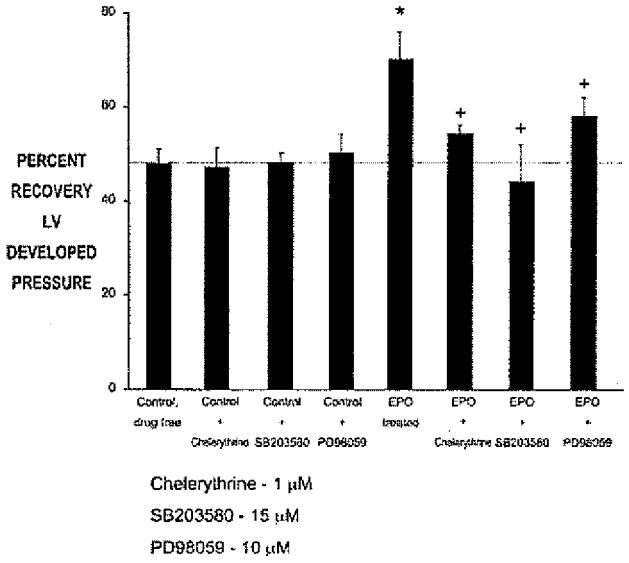

Fig. 3 Protein kinase-mediated cardioprotective effects of erythropoietin in infant rabbit hearts. Recovery of left ventricular developed pressure following 15 min treatment with erythropoietin (1.0 U/ml) and protein kinase inhibitors prior to 30 min ischemia and 35 min reperfusion. Data are means ± SD ($n = 8$ hearts/group). * = $P < 0.05$, EPO vs drug free control, + = $P < 0.05$ EPO + drug vs EPO

ulate fractions revealed that in erythropoietin-treated hearts, PKC_ε was activated and translocated from the cytosolic to the particulate fraction. Activation of PKC_ε occurs as early as 5 minutes after treatment with erythropoietin and remains active for as long as 15 minutes after treatment. Erythropoietin treatment for 5 minutes resulted in phosphorylation of p38 MAP kinase in the cytosolic fraction but not in the particulate fraction. However the extent of phosphorylation of p38 MAP kinase declined after 15 minutes treatment with erythropoietin. We detected minimal autophosphorylation of p38 MAP kinase in the cytosolic fraction of untreated hearts. Erythropoietin treatment for 5 minutes resulted in remarkable phosphorylation of p42/44 MAP kinase in the cytosolic fraction with minor phosphorylation in the particulate fraction. In contrast, treatment of hearts with erythropoietin for 15 minutes resulted in enhanced phosphorylation of p42/44 MAP kinase in the cytosolic fraction and in the particulate fraction (Fig. 4). Thus, the Western analysis studies confirm the functional recovery studies with inhibitors of protein kinases. Taken together our results indicate the acute cardioprotective effects of erythropoietin are mediated by activation of protein kinase signaling pathways.

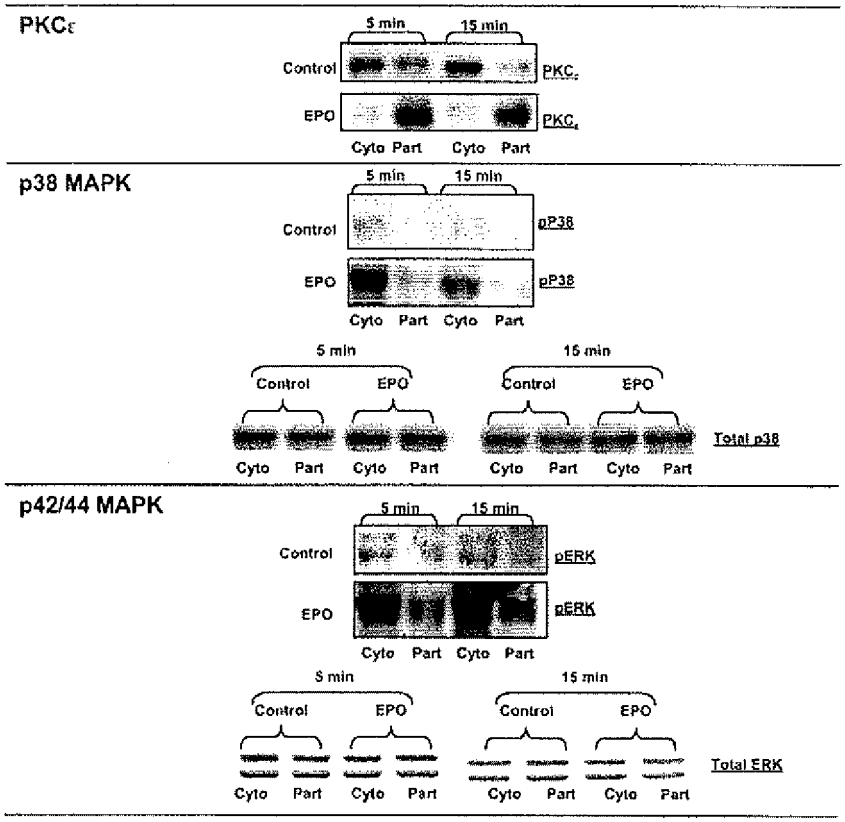

Fig. 4 Cardioprotection by erythropoietin-involvement of protein kinases. Western analysis of total cell lysates, cytosolic (cyto) and particulate (part) fractions of hearts treated with 1.0 U/ml erythropoietin (EPO) for 5 minutes or 15 minutes. Data are representative of three blots for each antibody

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Role of potassium channels in erythropoietin-induced cardioprotection

K_{ATP} channels, highly expressed in myocardial sarcolemma and thought to be expressed in myocardial mitochondria, have been found to serve as mediators of cardioprotection [7, 11, 15]. To investigate a role for K_{ATP} channels in mediating erythropoietin-induced cardioprotection, we performed the following studies in normoxic rabbits. Hearts were perfused with K_{ATP} channel blockers alone for 15 minutes and then in combination with erythropoietin (1.0 U/ml) for another 15 minute period prior to ischemia. Glibenclamide (3 μM), a non-specific K_{ATP} channel blocker, completely abolished the cardioprotective effect of erythropoietin (Fig. 5). The mitochondrial K_{ATP} channel blocker 5-hydroxydecanoate (300 μM) partially and the sarcolemmal K_{ATP} channel blocker HMR 1098 (30 μM) completely blocked the cardioprotective effects of erythropoietin. Thus the cardioprotective effects of erythropoietin are mediated by the sarcolemmal K_{ATP} channel with a possible additional role for the mitochondrial channel.

Recently another potassium channel, located in the inner mitochondrial membrane, the calcium-activated potassium (KCa) channel has been shown to mediate protection of the heart against ischemia [26]. We determined whether the mitochondrial KCa channel mediates the cardioprotective effects of erythropoietin. Hearts were perfused with Paxilline (1 μM), a blocker of the KCa channel, alone for 15 minutes and then in combination with erythropoietin (1.0 U/ml) for another 15 minute period prior to ischemia. Paxilline completely blocked the cardioprotective effect of erythropoietin but had no effect on untreated hearts. Our data suggest the cardioprotective effects of erythropoietin are mediated by the mitochondrial KCa channel (Fig. 5).

Role of nitric oxide synthase in erythropoietin-induced cardioprotection

Increased nitric oxide production from nitric oxide synthase serves to protect the heart against ischemic injury. As nitric oxide synthase has also been reported to mediate the cellular effect of erythropoietin [24], we determined if inhibition of nitric oxide synthase would affect cardioprotection induced with erythropoietin. Hearts from normoxic rabbits were perfused with nitric oxide synthase inhibitors combined with erythropoietin (1.0 U/ml) for 15 minutes prior to ischemia. L-NAME (200 μM) or L-NMA (100 μM) did not block the cardioprotective effect of erythropoietin (Fig. 6). Nitrite and nitrate release from hearts before (2.3 ± 0.9 nmoles/min/g) and after (2.4 ± 1.9 nmoles/min/g) 15 minutes treatment with erythropoietin (1.0 U/ml, $n = 8$) were not different. Our data suggest that nitric oxide synthase does not play a major role in mediating the cardioprotective effects of erythropoietin in this model.

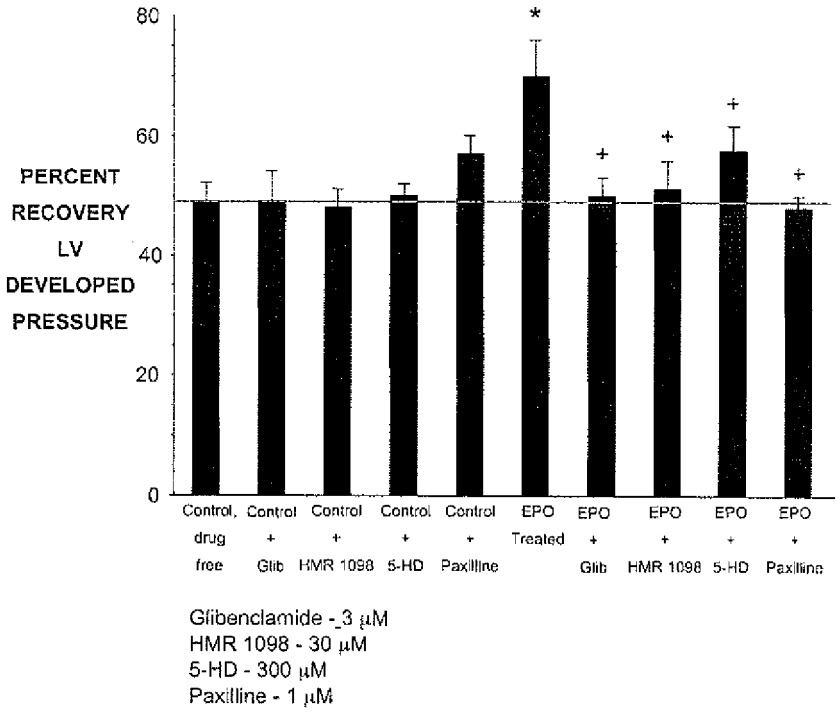

Fig. 5 Potassium channel-mediated cardioprotective effects of erythropoietin. Recovery of left ventricular developed pressure following 15 min treatment with erythropoietin (1.0 U/ml) and potassium channel blockers prior to 30 min ischemia and 35 min reperfusion. Data are means ± SD ($n = 8$/group). * = $P < 0.05$, EPO vs drug free control. + = $P < 0.05$, EPO + drug vs EPO

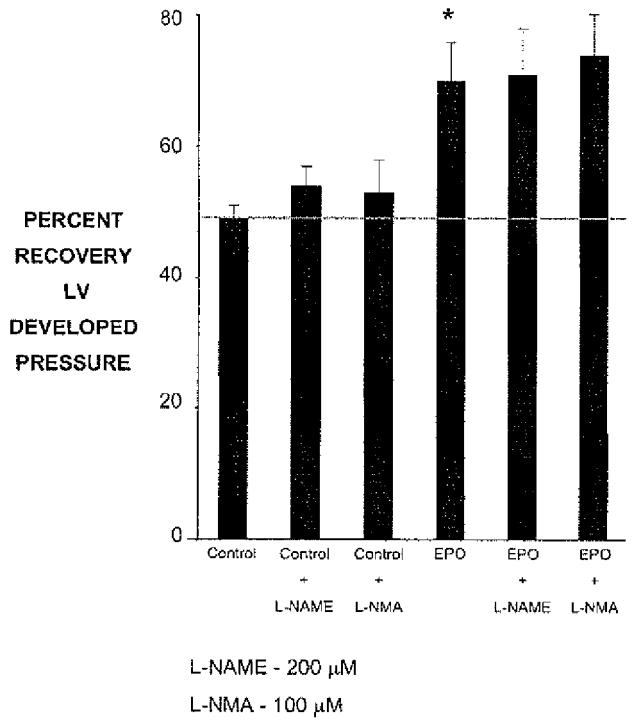

Fig. 6 Cardioprotective effects of erythropoietin: Role of nitric oxide synthase. Recovery of left ventricular developed pressure following 15 min treatment with erythropoietin (1.0 U/ml) and nitric oxide synthase inhibitors prior to 30 min ischemia and 35 min reperfusion. Data are means ± SD (n = 8/group). * = $P < 0.05$, EPO vs drug free control

Cardioprotection by erythropoietin in chronically hypoxic hearts

Adaptation to the stress of chronic hypoxia from birth to 10 days of age results in erythropoiesis and also increases resistance to myocardial ischemic injury [2]. We reasoned it would be important to determine whether erythropoietin confers cardioprotection in chronically hypoxic hearts since many children who have congenital heart defects exhibit varying degrees of cyanosis where erythropoietin increases hematocrit and hemoglobin levels. Hearts from 10 day old chronically hypoxic rabbits were treated with erythropoietin at a concentration of 1.0 U/ml. Erythropoietin did not increase recovery of developed pressure in either the left or right ventricle (Fig. 7). Thus normoxic and chronically hypoxic hearts respond differently to erythropoietin treatment. Recovery of LVDP was increased by 43% in normoxic infant hearts from 49 ± 2% to 70 ± 6% following treatment with erythropoietin at the optimal dose of 1.0 U/ml; this recovery is comparable with cardioprotection conferred by adaptation to chronic hypoxia. However, erythropoietin treatment did not increase recovery of LVDP and RVDP in hypoxic hearts, suggesting chronically hypoxic hearts are already maximally protected against ischemia.

Time to activate protein kinases vs time needed to confer cardioprotection

Phosphorylation of p38 MAP kinase was maximized following 5 minutes treatment with erythropoietin, whereas phosphorylation of p42/44 MAP kinase and PKC_ε were maximal following 15 minutes treatment (Fig. 4). Other reports have shown that PKC-MAP kinase pathway is activated within minutes of stimulation and then rapidly declines [1, 8]. Our findings are consistent with these previous observations. Treatment of hearts for 5 minutes with erythropoietin at the optimal dose of 1.0 U/ml prior to ischemia increased the recovery of right ventricular developed pressure from 68 ± 7% to 82 ± 12%, but has no effect on the recovery of left ventricular developed pressure (52 ± 6% vs. 49 ± 2%). The minimum treatment period with erythropoietin needed to confer cardioprotection in both left and right ventricle was 15 minutes. Hearts were also treated for 20 minutes with erythropoietin (1.0 U/ml). However, there was no further increase in cardioprotection above that conferred following 15 min treatment. We did not evaluate treatment times longer than 20 minutes. A possible explanation for the difference in time to activate protein kinases vs the time needed to confer cardioprotection is that the cardioprotective effect of activation of either of these protein kinases is likely mediated by a downstream component

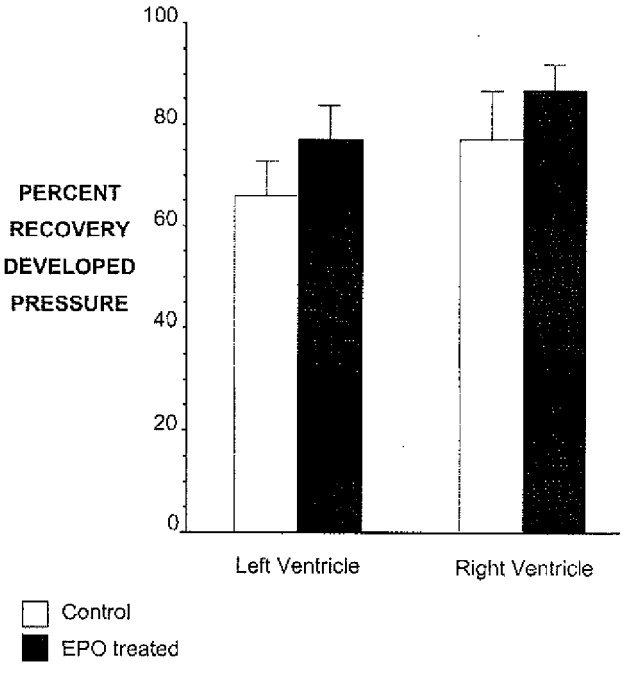

Fig. 7 Cardioprotection by erythropoietin in chronically hypoxic hearts. Recovery of left and right ventricular developed pressure following 15 min treatment with erythropoietin (1.0 U/ml) prior to 30 min ischemia and 35 min reperfusion. Data are means ± SD ($n = 8$/group)

(for example potassium channels) but not by the kinase *per se*. Thus 5 minutes of treatment with erythropoietin is sufficient to activate protein kinases, but insufficient to trigger subsequent downstream components that confer cardioprotection.

Circulating erythropoietin levels in infant rabbits

To compare the level of erythropoietin that confers optimal cardioprotection (1.0 U/ml) with the levels present in the circulation, we determined serum levels of erythropoietin. Serum levels of erythropoietin in normoxic and chronically hypoxic infant rabbits were 2.1 ± 0.4 mU/ml and 7.7 ± 4.0 mU/ml, respectively.

Discussion

Our study shows that erythropoietin acutely exerts a concentration- and time-dependent cardioprotective effect. The mechanisms underlying erythropoietin-induced cardioprotection involves activation of PKC_ε, p38 MAP kinase and p42/44 MAP kinase with increased resistance to myocardial ischemia mediated by potassium channels but not by nitric oxide synthase. The optimal concentration of 1.0 U/ml needed to confer protection against cardiac ischemia is approximately 100 times above levels present during chronic hypoxia and 500 times above erythropoietin levels present in the circulation of normoxic rabbits. Increased resistance to myocardial ischemia is observed immediately after treatment with erythropoietin, indicating that induction of new genes is not necessary for its cardioprotective effect to be manifested. We believe our study is the first to demonstrate the biological effects of erythropoietin are mediated by a signal pathway that results in acute activation of two potassium channels, the K_{ATP} and the KCa channel. Importantly, protection by erythropoietin is redundant of the cardioprotective effects of chronic hypoxia. Activation of the p38 MAP kinase pathway is responsible for increased cardioprotection in the chronically hypoxic heart [17]. Our study shows that erythropoietin induces activation of the MAP kinase pathway in the myocardium and also involves a unique and strong activation of PKC.

Several distinct types of potassium channel are present in heart, of which two, the K_{ATP} and the KCa channel serve to protect the heart against conditions of oxygen deprivation, such as hypoxia and ischemia. However the underlying mechanisms remain to be established. We show that erythropoietin-induced protection against ischemia is completely prevented by glibenclamide, a non-specific K_{ATP} channel blocker and by HMR 1098, a sarcolemmal specific K_{ATP} channel blocker. In contrast, 5-HD, a blocker of the mitochondrial K_{ATP} channel only partially prevented the cardioprotective effects of erythropoietin. Furthermore, paxilline a blocker of both sarcolemmal and mitochondrial KCa channels completely abrogated the protection provided by erythropoietin. The sarcolemmal K_{ATP} channel and the sarcolemmal and mitochondrial KCa channels appear to play a pivotal role with a partial involvement of the mitochondrial K_{ATP} channel in erythropoietin-induced cardioprotection [12, 18]. These potassium channels are thought to be located at two sites within the cell, the sarcolemma and the mitochondria. Once activated sarcolemmal K_{ATP} and KCa channels promote potassium efflux from the cytosol to outside the cell, while activation of mitochondrial K_{ATP} and KCa channels result in an influx of potassium from the cytosol into the mitochondria. Activation of sarcolemmal K_{ATP} and KCa channels may act to reduce calcium influx into the cell during ischemia. In addition, the sarcolemmal K_{ATP} channel may also be responsible for opening the mitochondrial K_{ATP} channel. In contrast, activation of mitochondrial K_{ATP} and KCa channels may mediate cardioprotection by improved energetics [10, 26].

The limitations of our study are that we could not identify the cell type in which PKC_ε and MAP kinases are activated and that contain the K_{ATP} and KCa channels. The proposed role of protein kinases and potassium channels in the signal transduction pathway by which erythropoietin increases the resistance of the infant heart to ischemia have been based on experiments with kinase inhibitors and potassium channel blockers applied at conventional inhibitory concentrations. This pharmacological approach is dependent on the relative specificity of the inhibitors and blockers used. For example the role of the sarcolemmal K_{ATP} channel in erythropoietin-induced cardioprotection is based on pharmacological studies with HMR 1098, a blocker of this channel. The specificity of this blocker has recently been questioned as HMR 1098 abolishes the cardioprotective effect of diazoxide, an opener of the mitochondrial K_{ATP} channel [23]. However, HMR 1098 has no effect on the activity of reconstituted mitochondrial K_{ATP} channels [27]. The cardioprotective effect of erythropoietin is due in part to activation of mitochondria KCa channels located in the cardiomyocytes [10, 26]. However, this channel may exist in other locations in the heart such as the sarcolemma and may exert its effect on other KCa channels such as those present in the cardiac nerves and smooth muscle cells.

Our study indicates that erythropoietin confers cardioprotection by a mechanism that does not appear to involve nitric oxide synthase. This finding contrasts with other studies where erythropoietin (20 U/ml) stimulates nitric oxide release [25] from endothelial cells. Comparison of the experimental protocol between the two studies reveals that chronic treatment with high concentrations of erythropoietin were needed to stimulate nitric

oxide release, whereas in our study the optimal concentration of erythropoietin is far lower and does not result in erythropoiesis. Thus our studies reveal a novel non-erythropoietic action of erythropoietin that is manifested acutely at pharmacologic levels.

The level of cardioprotection achieved with erythropoietin is comparable to that conferred by ischemic preconditioning [4]. Ischemic preconditioning is a powerful endogenous phenomenon in which brief episodes of a subtoxic ischemic insult induces robust protection against more prolonged, lethal ischemia. The molecular mechanisms underlying ischemic preconditioning are still being elucidated. Clinical application of ischemic preconditioning remains elusive and has not yet gained widespread acceptance as a treatment strategy. Pharmacologic preconditioning against ischemia may offer a more practical way of harnessing the molecular mechanisms responsible for increased cardioprotection. We have shown that pharmacological preconditioning through erythropoietin is effective and represents a novel cardioprotective strategy in the setting of elective myocardial ischemia as encountered during cardiac surgery and angioplasty. Erythropoietin is currently approved and available for human clinical use. This well-tolerated compound does not require an elaborate drug delivery system as is needed for many gene-based therapies.

Erythropoietin has been suggested to be a mediator of ischemic preconditioning in the brain since it is produced after lethal ischemic or hypoxic insults [9, 20].

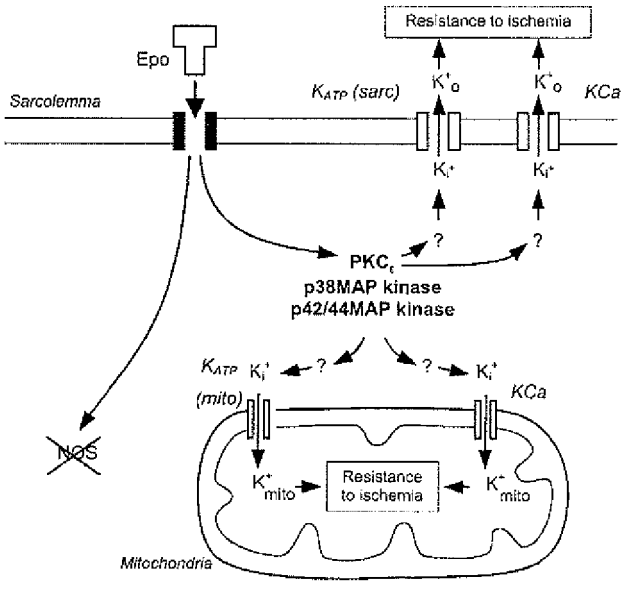

Fig. 8 Schematic representation of signaling pathways by which erythropoietin may confer acute cardioprotection

Recently an elegant study by Calvillo et al. [6] demonstrated erythropoietin protects adult myocardium against ischemia in both *in vivo* and *in vitro* models. Our study in infant rabbit myocardium complements their study and additionally provides mechanistic data on signaling pathways associated with cardioprotection by erythropoietin. Neural expression of erythropoietin is actually reduced after stimuli that induce ischemic preconditioning in the brain [14]. In addition, lethal stresses and hypoxia/ischemia clearly induce erythropoietin, but sublethal preconditioning stimuli may not be potent enough to produce substantial concentrations of erythropoietin [14]. Thus, erythropoietin may be a suitable exogenous agent to pharmacologically precondition the heart against ischemia. To confer cardioprotection erythropoietin must be given before the ischemic insult. Thus planned ischemic events such as cardiac surgery, angioplasty or preservation of donor hearts for transplantation represent an opportunity to harness the cardioprotective effects of erythropoietin.

The optimal dose in our study to confer cardioprotection is 1.0 U/ml. In a cerebral model of ischemic injury, for *in vivo* studies a dose of erythropoietin at 5,000 U/kg conferred protection [21]; whereas for our *in vitro* studies erythropoietin at 0.5 – 5.0 units/ml was protective. Erythropoietin (5,000 U/kg) has been used to confer delayed cardioprotection in the rat and rabbit increasing post ischemic function recovery [16] and decreasing apoptosis [5] and by reducing infarct size [16]. Thus the concentration of erythropoietin required to confer protection against ischemia is comparable for both brain and heart. Our study expands on these recent observations by providing mechanistic information on protein kinase signal transduction pathways and potassium channels mediating cardioprotection by erythropoietin. We have included a representation of the signaling pathway by which erythropoietin may confer acute cardioprotection (Fig. 8).

Erythropoietin increases resistance to ischemia in normoxic hearts. Thus the greatest benefit would be to normoxic infants undergoing cardiac surgery for repair of congenital heart defects. In contrast, hearts adapted to severe chronic hypoxia already exhibit increased resistance to ischemia compared with normoxic hearts [2]. We showed that erythropoietin does not further increase the level of cardioprotection in chronically hypoxic hearts, indicating cardioprotection by erythropoietin is redundant in these hearts. Furthermore, erythropoietin does not appear to exert any adverse effect on ischemic myocardium in chronically hypoxic infants. In human infants with cyanotic heart defects chronic hypoxia may be intermittent or continuous in nature with the myocardium exposed to varying degrees of hypoxia. Thus erythropoietin may also be able to confer cardioprotection in infants with mild degrees of hypoxia.

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